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Form PTO-1390 (REV 11-98)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 410718.90395
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371			
INTERNATIONAL APPLICATION NO. PCT/CA00/01132	INTERNATIONAL FILING DATE 21 Sept 2000 (21.09.00)	PRIORITY DATE CLAIMED 21 Sept 1999 (21.09.99)	
TITLE OF INVENTION LOCAL DELIVERY OF 17-BETA ESTRADIOL FOR PREVENTING VASCULAR INTIMA HYPERPLASIA AND FOR IMPROVING VASCULAR ENDOTHELIUM FUNCTION AFTER VASCULAR INJURY			
APPLICANT(S) FOR DO/EO/US CHANDRASEKAR, Baskaran; TANGUAY, Jean-Francois			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
<ol style="list-style-type: none">1. <input checked="" type="checkbox"/> This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.2. <input type="checkbox"/> This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.3. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))<ol style="list-style-type: none">a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US)6. <input type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))<ol style="list-style-type: none">a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).b. <input checked="" type="checkbox"/> have been transmitted by the International Bureau.c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.d. <input type="checkbox"/> have not been made and will not be made.8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).			
Items 11. to 16. below concern document(s) or information included:			
11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98 and Form 1449.			
12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.			
13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.			
14. <input type="checkbox"/> A substitute specification.			
15. <input type="checkbox"/> A change of power of attorney and/or address letter.			
16. <input checked="" type="checkbox"/> Other items or information: Copy of Form PCT/IB/308 dated 29 March 2001 Confirming Transmittal of the International Application to the US as Designated Office; Postcard			

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.1)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER		
10/088405	PCT/CA00/01132	410718.90395		
17. [X] The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):		CALCULATIONS PTO USE ONLY		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1040.00				
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$890.00				
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$740.00				
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$710.00				
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00				
ENTER APPROPRIATE BASIC FEE AMOUNT =		\$890.00		
Surcharge of \$130.00 for furnishing the oath or declaration later than [] 20 [] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		\$		
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	8	-20 =	0	X \$18.00
Independent claims	1	-3 =	0	X \$78.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)		+ \$260.00	\$	
TOTAL OF ABOVE CALCULATIONS =		\$890.00		
[X] Applicant hereby claims small entity status. Reduction by 1/2 for filing by small entity.		\$445.00		
SUBTOTAL =		\$445.00		
Processing fee of \$130.00 for furnishing the English translation later than [] 20 [] 30 months from the earliest claimed priority date (37 CFR 1.429(f)).		+ \$		
TOTAL NATIONAL FEE =		\$445.00		
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property		+ \$		
TOTAL FEES ENCLOSED =		\$445.00		
		Amount to be: refunded	\$	
		Charged	\$	
a. [] A check in the amount of <u>\$.00</u> to cover the above fees is enclosed.				
b. [X] Please charge my Deposit Account No. <u>17-0055</u> in the amount of <u>\$445.00</u> to cover the above fees. A duplicate copy of this sheet is enclosed.				
c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>17-0055</u> . A duplicate copy of this sheet is enclosed.				
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.				
SEND ALL CORRESPONDENCE TO:				
 Jean C. Baker SIGNATURE <hr/> Jean C. Baker NAME <hr/> 35,433 REGISTRATION NUMBER				
Quarles & Brady LLP 411 East Wisconsin Ave. Milwaukee, WI 53202-4497				

10/088405

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: **CHANDRASEKAR** Docket No.: 410718.90395

Serial No.: **Unassigned** Filed: **Concurrently herewith**

Int'l appln No.: **PCT/CA00/01132** Int'l filing date: **21 Sept 2000**

Title: **LOCAL DELIVERY OF 17-BETA ESTRADIOL FOR
PREVENTING VASCULAR INTIMA HYPERPLASIA AND FOR
IMPROVING VASCULAR ENDOTHELIUM FUNCTION AFTER
VASCULAR INJURY**

PRELIMINARY AMENDMENT

Box PCT
Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

In connection with the above-identified application filed herewith, please enter the following preliminary amendment:

IN THE CLAIMS:

The claims have been amended to read as follows. A copy of the marked claims showing the amendments made is attached.

1. The use of 17-β estradiol or a derivative thereof in the making of a medication or a device for in-situ administration in the lumen of a blood vessel having suffered vascular injury, at the injured site, for improving reendothelialization and vascular endothelial function in a patient.

2. The use as defined in claim 1, wherein 17- β estradiol or a derivative thereof is present in a dose unit of 1 to 5000 $\mu\text{g}/\text{Kg}$ of patient's body weight.
3. The use, as defined in claim 1, wherein 17- β estradiol or a derivative thereof is present in a dose unit of 10 to 50 $\mu\text{g}/\text{Kg}$ of patient's body weight.
4. The use as defined in claim 1, wherein 17- β estradiol or a derivative thereof is present in a dose unit of 10 to 30 $\mu\text{g}/\text{Kg}$ of patient's body weight.
5. The use as defined in claim 1, wherein said pharmaceutically acceptable carrier comprises hydroxypropyl-beta-cyclodextrin (HPCD).
6. The use as defined in claim 5, wherein HPCD is present in a dose capable of solubilizing 17-beta estradiol or a derivative thereof.
7. The use as defined in claim 4, wherein 17-beta-estradiol or a derivative thereof is admixed with a carrier comprising at least 0.63 mg hydroxypropyl-beta-cyclodextrin per kilogram of patient's body weight.
8. The use as defined in claim 1, which is for a single administration.

Remarks

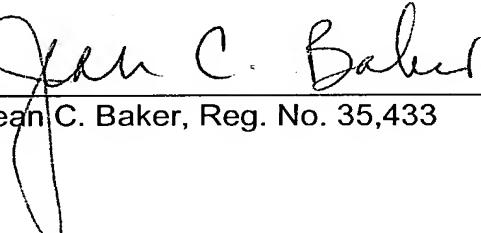
The above amendments are being made to eliminate multiple dependencies in the claims of this application.

No fee is believed necessary to enter this amendment. However if a fee is necessary, please charge Deposit Account 17-0055.

Applicant respectfully requests that the preliminary amendment described herein be entered into the record prior to examination and consideration of the above-identified application.

QUARLES & BRADY LLP

BY:


Jean C. Baker, Reg. No. 35,433

Date: March 18, 2002

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: **CHANDRASEKARr** Docket No.: 410718.90395
Serial No.: **Unassigned** Filed: **Concurrently herewith**
Int'l appln No.: **PCT/CA00/01132** Int'l filing date: **21 Sept 2000**
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CLAIM SET SHOWING AMENDMENTS MADE

1. The use of 17- β estradiol or a derivative thereof in the making of a medication or a device for in-situ administration in the lumen of a blood vessel having suffered vascular injury, at the injured site, for improving reendothelialization and vascular endothelial function in a patient.
2. The use as defined in claim 1, wherein 17- β estradiol or a derivative thereof is present in a dose unit of 1 to 5000 μ p/Kg of patient's body weight.
3. The use, as defined in claim 1, wherein 17- β estradiol or a derivative thereof is present in a dose unit of 10 to 50 μ p/Kg of patient's body weight.
4. The use as defined in claim 1, wherein 17- β estradiol or a derivative thereof is present in a dose unit of 10 to 30 μ p/Kg of patient's body weight.
5. The use as defined in [any one of claims 1 to 5] claim 1, wherein said pharmaceutically acceptable carrier comprises hydroxypropyl-beta-cyclodextrin (HPCD).

6. The use as defined in claim 5, wherein HPCD is present in a dose capable of solubilizing 17-beta estradiol or a derivative thereof.

7. The use as defined in claim 4, wherein 17-beta-estradiol or a derivative thereof is admixed with a carrier comprising at least 0.63 mg hydroxypropyl-beta-cyclodextrin per kilogram of patient's body weight.

8. The use as defined in [any one of claims 1 to 7] claim 1, which is for a single administration.

TITLE OF THE INVENTION

Local Delivery of 17-beta Estradiol for Preventing vascular
intimal hyperplasia and for improving vascular endothelium function after
5 vascular injury

FIELD OF THE INVENTION

The present invention relates to the local use of estradiol or
10 a derivative thereof to improve the outcome of a coronary angioplasty. More
specifically, the present invention is concerned with the local use of estradiol
or a derivative thereof for decreasing neointima hyperplasia that occurs
during restenosis, and for improving the endothelium function after vascular
injury, both events contributing to the ultimate success of an angioplasty.

15

BACKGROUND OF THE INVENTION

Restenosis is currently the major limitation of percutaneous
transluminal coronary angioplasty (PTCA), and is seen in up to 30-40 % of
20 patients.¹ The most important mechanisms contributing to restenosis are
neointima proliferation, vascular remodelling, and elastic recoil.² Elastic recoil
and vascular remodelling can be reduced to a large extent by stenting.³
Although radiation therapy has been reported to show beneficial effects,^{4,5}
no effective therapy exists yet for neointima proliferation. Vascular smooth
25 muscle cell (SMC) migration and proliferation have been documented to
occur as early as 36 hours following arterial injury.⁶ In cell culture assays, 17-
beta estradiol inhibited migration and proliferation of rat vascular SMC.^{7,8}
Similar effects have also been shown with human vascular SMC from
saphenous vein.⁹ Prolonged systemic administration of estrogen has been
30 shown to inhibit intima hyperplasia in animal studies.^{10,11} Instead of

administering estradiol systematically we here tested how a local administration of 17-beta estradiol during PTCA could effectively inhibit neointima proliferation.

5 The vital role of endothelium in the regulation of vascular tone of arteries is well-recognized (1). The intact endothelium also has important inhibitory effects on platelet aggregation, monocyte adhesion, and vascular smooth muscle cell proliferation (2). Endothelial injury associated with endothelial dysfunction is known to occur as a consequence of
10 percutaneous transluminal coronary angioplasty (PTCA) (3), and may play an important role in restenosis following PTCA (4). Impaired endothelial function has been demonstrated in porcine coronary arteries as long as 4 weeks following PTCA in pigs (5). Systemically administered 17-beta estradiol has been reported to accelerate endothelial recovery after arterial
15 injury (10). Since endothelial injury due to PTCA is a local event, we hypothesized that local delivery of 17-beta estradiol following PTCA may enhance endothelial recovery.

SUMMARY OF THE INVENTION

20 An object of the present invention is therefore to provide efficient methods by which 17-β estradiol or a derivative thereof is used locally during PTCA to improve endothelial function after vascular injury and/or to decrease the neointima hyperplasia and/or prevent restenosis. Compositions for executing
25 these methods are also a further object of this invention.

Other objects, advantages and features of the present invention will become more apparent upon reading of the following nonrestrictive description of preferred embodiments thereof, given by way of
30 examples only, with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 Representative light micrographs (x 40 magnification) of arterial segments from the same animal, stained with Verhoeff's stain. 17-beta estradiol (a) treated segment shows markedly less neointima hyperplasia compared to PTCA only (b), or vehicle alone (c) groups. The extent of injury is similar in all 3 segments.

Figure 2 Comparison of (A) neointima area, (B) neointima/media area, (C) restenotic index, and (D) % stenosis between PTCA alone vs vehicle only, and PTCA only vs 17-beta estradiol groups; * p < 0.05, ** p < 0.01 *** p < 0.002. Values are expressed as mean ± SEM.

Figure 3 Representative coronary angiograms demonstrating the vasoconstrictive response to intracoronary infusion of acetylcholine (Ach) 10⁻⁴M obtained from the same animal at 4 weeks following percutaneous transluminal coronary angioplasty (PTCA). Column A = basal, column B = after Ach, column C = following intracoronary nitroglycerin. Top panel = treatment with vehicle, mid panel = PTCA only, lower panel 17-beta estradiol treatment groups respectively.

Figure 4 Representative light micrographs (x 1000) of cross sections of vessels obtained from the same animal for immunohistochemical staining with the lectin *Dolichos biflorus* agglutinin (evident as dark brown staining of luminal surface). Vessels treated with 17-beta estradiol (A) demonstrate reendothelialization to a greater degree as compared to PTCA only (B) and vehicle (C) groups.

Figure 5 Representative light micrographs (x 1000) of cross sections of vessels obtained from the same animal, for immunohistochemical analysis

of endothelial nitric oxide synthase (eNOS) expression. Vessels treated with 17-beta estradiol (A) show greater expression of eNOS (evident as dark brown staining of luminal surface) as compared to PTCA only (B) and vehicle (C) groups.

5

Figure 6 Graph depicting correlation between vasoconstrictive response to Ach 10^{-4} M and (A) reendothelialization ($r = -0.48$, $p < 0.02$), (B) eNOS expression ($r = -0.58$, $p < 0.005$). Note: % vasoconstriction denotes % decrease in diameter following Ach 10^{-4} M as compared to the basal 10 diameter.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Example 1: The effect of estradiol on neointima hyperplasia

15 **Methods**

Animal preparation

Eighteen juvenile farm pigs (9 female, and 9 castrated male) weighing 20-25 kg were studied. The study was approved by, and conducted in accordance 20 with, the guidelines of the Animal Care and Ethical Research Committee of the Montreal Heart Institute. Before the procedure, animals were given 650 mg of acetylsalicylic acid and 30 mg of nifedipine orally, premedicated with intramuscular injection of 6 mg/kg of a mixture of tiletamine hydrochloride and zolazepam hydrochloride, and given 0.05 mg of atropine. The invasive 25 procedure was performed under general anesthesia with a mixture of isoflurane (1 to 1.5 %) and oxygen enriched air. The right femoral artery was cannulated percutaneously, and an 8 Fr arterial sheath was introduced. After arterial access had been obtained, 100 mg of lidocaine and 250 U/kg of heparin were administered intra-arterially via the sheath. Activated

coagulation time was maintained at > 300 seconds throughout the procedure.

Preparation of estradiol formulation

5

Each dose individually administered to the tested animals is composed of at least 12.5 mg hydroxypropyl-beta-cyclodextrin (HPCD) and 600 µg estradiol in a 5 ml solution volume.

10 A smaller or larger dose may be used. Indeed, the tested dose corresponds to the dose of about 675 µg formulated in a sublingual pellet and administered to postmenopausal women.⁴⁵ Such a dose may be unnecessarily high if administered locally. Indeed, doses of 200 and 400 µg have been tried and they were found to be as performing as the dose of
15 600 µg. Further, the necessary dose for performing the present invention may be influenced by the hormonal balance of the individual to be treated. Species variance is also a factor affecting the dosage regimen. Also, any derivative of 17-beta estradiol may replace the latter. A derivative is intended to cover a precursor, an active metabolite, an active analog or a
20 modulator capable of positively influencing the activity of the receptor(s) to estradiol or of enhancing the binding and/or the activity of estradiol towards its receptor(s). Such derivatives are considered functional equivalents of 17-beta-estradiol, and therefore within the scope of this invention. A unit dose of 1 to 5000 µg/Kg of 17-beta-estradiol or an equivalent derivative dose is
25 within the scope of this invention, preferably 10-50 µg/Kg, even more preferably 10-30 µg/Kg.

Angioplasty and Local Delivery

Standard PTCA equipment was used. An 8 Fr right Amplatz guiding catheter and right Judkins guiding catheter were used for cannulation of the left and 5 right coronary arteries, respectively. PTCA was performed with a balloon size chosen to correspond to a balloon/artery ratio of 1.1-1.3. Three 30-second inflations at 10 atm pressure were performed with a 30-second interval between each inflation. Inflations were performed adjacent to major side branches to facilitate identification during harvesting, taking precaution not 10 to include any side branch in the intended PTCA site. The left anterior descending, left circumflex, and right coronary arteries of each animal were subjected to PTCA. After PTCA, each coronary artery of an animal was randomized to receive either 600 µg of 17-beta estradiol locally, or vehicle alone locally, or PTCA only. The chemicals 17-beta estradiol and its vehicle 15 2-hydroxypropyl-beta-cyclodextrin (HPCD) were purchased from Sigma Chemical Co. The InfusaSleeve catheter (Local Med, Inc.) was used for local delivery.¹² Five ml of the designated substance was delivered at a driving pressure of 10 atm and support balloon pressure of 6 atm.

20 Of the 18 animals, 2 died a few days after PTCA, and were excluded; thus, 16 animals were analyzed. Twelve animals were euthanized at 28 days, and 4 at 7 days. After premedication and anesthesia, the right internal jugular vein and common carotid artery were cannulated. Following cross-clamping of the descending thoracic aorta exposed via a left lateral thoracotomy, 25 exsanguination was performed, with simultaneous administration of 1 l of 0.9 % NaCl solution. The heart was perfusion-fixed in vivo with 2 l of 10 % buffered formalin at 200 mm Hg pressure, removed from the animal, and placed in 10 % buffered formalin solution. Coronary arteries were then dissected free from surrounding tissues. The site of PTCA was identified in 30 relation to adjacent side branches, which served as landmarks. The injured

segment was harvested with a 1 cm normal segment proximal and distal to the injured site. Serial sections 3 to 5 mm long were made from the harvested segment, with a minimum of at least 3 sections (maximum 5) from each PTCA site. Sections were stored in buffered 10 % formalin and
5 subjected to dehydration with increasing concentrations of alcohol, followed by treatment with xylene and paraffin. Each section was then cut to slices of 6 μ m thickness with a microtome (Olympus cut 4060 E), and stained with Verhoeff's stain for morphometric analysis.

10 Morphometric analysis

Measurements were made with a video microscope (Leitz Diaplan, equipped with a Sony DXC 970 MD color video camera) linked to a 486 personal computer and customized software. A minimum of 3 sections for each injured
15 segment were analyzed and results averaged. Analyses were made by a single observer unaware of the treatment group to which each segment had been allocated. Randomly selected sections were viewed by a second observer (also blinded to protocol) independently; inter-observer variability was < 5 %. The areas of external elastic lamina (EEL), internal elastic lamina (IEL), and lumen were measured by digital planimetry; neointima (I) area (IEL - lumen area) and media (M) area (EEL - IEL area) were obtained. The % neointima was defined as the % of total vessel area occupied by neointima (% neointima = [I/EEL] \times 100). Morphologic % stenosis was calculated as 100 (1 - lumen/IEL area).¹³ The restenotic index was defined as
20 [I/(I + M)]/(F/IEL circumference), where F is the fracture length of internal elastic lamina.¹⁴ Histologic injury score was determined as previously defined.¹⁵

Immunohistochemistry

Following slicing with a microtome and blocking of non-specific antibodies, the sections were treated with mouse anti-proliferating cell nuclear antigen (PCNA) antibodies and diluted biotinylated goat anti-mouse antibodies. They were then incubated with avidin-biotin (Elite ABC Kit, Vector Laboratories), and developed with 3, 3'-diaminobenzidine (Vector Laboratories). They were finally counter-stained with hematoxylin. Porcine liver cells were used as a positive control. For each section, a 6 µm slice counter-stained with hematoxylin without treatment with the primary antibody (mouse anti- PCNA) served as a negative control.

The proliferative response to injury was studied by immunohistochemical analysis of samples from animals euthanized at 7 days. The % proliferating SMC was obtained by dividing the number of PCNA - positive SMC by the total number of SMC in each field; separate measurements were made for neointima and media layers. The proliferating cells were identified as SMC by positive staining of parallel sections with a smooth muscle actin antibody. To standardize comparison among treatment groups, measurements were obtained at 4 fixed locations separated by 90° sites for each section, and the results averaged. For each segment, two sections demonstrating maximal neointima response were analyzed, and the results averaged.

Statistical Analysis

Values are expressed as mean ± standard deviation, except as otherwise indicated. Kruskal - Wallis analysis was used for comparison of data among the 3 groups; subsequently, 17-beta estradiol and vehicle alone groups were separately compared with the PTCA only group using the Mann - Whitney rank sum test. Chi - square analysis was used for comparison of proportions.

The Mann - Whitney rank sum test was also used for comparison of data between male and female animals within the 17-beta estradiol treated group. Values were considered statistically significant if $p < 0.05$.

5 Results

Following PTCA and local delivery, animals were allowed to recover, and gained weight steadily. Two animals died 48 and 72 hours after procedure respectively, and were not included; thus 16 animals were studied. Autopsy 10 of the 2 animals revealed occlusive thrombus at the site of PTCA (in the 17-beta estradiol treated vessel in one pig, and in the vessel treated with PTCA only in the other pig).

Injured segments

15 Balloon/artery ratio and artery diameter were not significantly different among the 3 treatment groups (Table 1). Segments with intact IEL in which discernible injury was absent were excluded from analysis (2 from PTCA only group, and 1 from vehicle alone group). Two segments were lost during 20 harvesting and processing (1 of vehicle alone, and 1 of PTCA only group).

Morphometric analysis

Of the 12 animals that underwent morphometric analysis at 28 days, arterial 25 segments treated with local delivery of 17-beta estradiol showed significantly less neointima hyperplasia (Figure 1). This beneficial effect was noted in all parameters of neointima response to injury that were analyzed (Table 1). Of note, the extent of morphologic injury was similar among the 3 groups, suggesting that the use of the InfusaSleeve catheter was not associated with 30 an enhanced risk of injury.

It was important to exclude an inhibitory effect on intima proliferation due to the vehicle, and, to confirm that the effect noted was in response to treatment with 17-beta estradiol. Analyses comparing segments treated with 5 vehicle alone and PTCA only showed a similar response in terms of the extent of neointima proliferation. On the other hand, significantly less intima hyperplasia was observed in 17-beta estradiol treated segments as compared to segments treated with PTCA only (Figure 2). Compared to PTCA only, or vehicle alone, 17-beta estradiol decreased neointima 10 formation by 54.6 % and 64.9 % respectively.

To exclude the possibility of influence of sex on response to estrogen, the 7 segments obtained from male pigs treated with 17-beta estradiol, and 5 segments obtained from female pigs treated with 17-beta estradiol were 15 analyzed. No statistically significant differences were evident (Table 2).

Immunohistochemistry

The number of PCNA - positive SMC was low overall; sacrifice at an earlier 20 time might have yielded a higher number. However, a statistically significant decrease in the proliferative response was seen in animals treated with 17-beta estradiol. Among the different groups, the % of PCNA - positive SMC in the neointima were $0.43 \pm 0.52\%$ in 17-beta estradiol, $4.26 \pm 2.33\%$ in PTCA only, and $4.27 \pm 2.73\%$ in vehicle alone groups respectively ($p < 0.05$ 25 for 17-beta estradiol vs other 2 groups). There were no statistically significant differences in % PCNA - positive SMC in the media among the 3 groups: $0.4 \pm 0.3\%$, $1.38 \pm 1.74\%$, and $1.24 \pm 1.57\%$ for 17-beta estradiol, PTCA only, and vehicle alone groups respectively ($p = NS$).

Vascular remodeling

To determine the effect on vascular remodeling of the agents used, the EEL area of the injured segment and of the normal vessel proximal to site of PTCA were obtained, and their ratio calculated.¹³ No significant difference among the groups was noted: 1.01 ± 0.16, 1.16 ± 0.28, 1.31 ± 0.37 respectively for 17-beta estradiol, PTCA only, and vehicle alone groups respectively (p = NS).

10 Conclusions

The present study demonstrates, for the first time, that locally delivered 17-beta estradiol decreases neointima proliferation following PTCA in pigs. The study also shows that the InfusaSleeve catheter can be used to deliver effectively 17-beta estradiol intramurally in coronary arteries.

Several previous experiments in animals have demonstrated that estrogen administered subcutaneously for up to 3 weeks inhibited the myointima response to arterial injury.^{10,11} Recently, short-term subcutaneous estrogen therapy (6 to 17 days) was also shown to be effective in reducing the injury response in rat carotid artery.¹⁶ Estrogen administered intra-muscularly for at least 3 weeks has also demonstrated the potential to inhibit vascular smooth muscle cell proliferation and neointima hyperplasia in rabbits.¹⁷ However, the efficacy of local delivery of 17-beta estradiol to inhibit intima hyperplasia has not been previously studied.

The biologic effects of estrogen, like other steroid hormones, involve intracellular receptors. The first estrogen receptor (ER) to be discovered was ER α ,^{18,19} which was thought to mediate the beneficial effects of estrogen following vascular injury. ER α was also present in coronary arteries obtained

from autopsy specimens in both pre and postmenopausal women,²⁰ and in cell cultures of human saphenous vein and internal mammary artery specimens.²¹ Recently, a second estrogen receptor, ER β , has been identified in animals and humans.^{22,23} The role of ER β in response to vascular injury 5 was subsequently demonstrated in experiments with ER α deficient mice.²⁴ Normal and ER α deficient mice treated with estrogen, when subjected to arterial injury, showed the same extent of inhibition of neointima proliferation compared to control mice; thereby demonstrating that inhibition of vascular injury response by estrogen is independent of ER α . Although the present 10 experiment was not designed to study the mechanism of action of 17-beta estradiol, evidence exists for multiple potential mechanisms by which 17-beta estradiol can inhibit the vascular response to injury. Of importance may be the effect of 17-beta estradiol on nitric oxide (NO) synthesis. In cell culture studies with human and bovine endothelial cells, treatment with 17-beta 15 estradiol stimulated NO synthase and increased NO production.^{25,26} Postmenopausal women treated with transdermal 17-beta estradiol showed enhanced in vivo NO synthesis.²⁷ NO has demonstrated inhibitory effects on both migration²⁸ and proliferation²⁹ of vascular SMC, and decreased neointima formation after PTCA.¹³ Preliminary reports have shown that 20 therapy with 17-beta estradiol decreases intercellular and vascular cell adhesion molecule expression by human coronary SMC.³⁰ Cellular adhesion molecules are expressed by SMC following arterial injury³¹ and their suppression with the use of monoclonal antibodies inhibited intima hyperplasia after arterial injury in rats.³² The regulatory effect of 17-beta 25 estradiol on vascular endothelial growth factor expression may also be partly responsible.³³⁻³⁵ Perhaps the most important mechanism may be a direct inhibitory effect of 17-beta estradiol on vascular SMC proliferation.³⁶ The binding of 17-beta estradiol to its intracellular receptor activates DNA containing "estrogen responsive elements", leading to altered gene

expression. 17-beta estradiol also reduces platelet derived growth factor-induced migration and proliferation of vascular SMC.⁹

The beneficial effects of 17-beta estradiol, the predominant circulating estrogen in premenopausal women, on vascular injury response may not be replicated by other kinds of estrogens; for example, conjugated equine estrogen was found to have no effect on neointima proliferation in non-human primate models.³⁷ Simultaneous administration of progesterone may attenuate the vascular injury response to 17-beta estradiol.³⁸ A sexually dimorphic response to estrogen in intact rats has been reported following arterial injury, with male rats deriving no benefit with estrogen therapy.³⁹ This sexually dimorphic effect was, however, not observed in another experiment with gonadectomized rats.¹¹ In the present study, too, no significant difference in neointima proliferative response to 17-beta estradiol was noted between the sexes. Increased expression of ER β mRNA (ER β is directly associated with inhibition of vascular SMC proliferation) following arterial injury has been demonstrated in intact male rats;⁴⁰ of additional interest in the study is that no increase in ER α was seen following arterial injury.

17-beta estradiol is a lipophilic compound with poor solubility in aqueous solutions, thereby needing a vehicle for parenteral administration. HPCD is a starch derivative that has been successfully tested as an effective excipient for protein drugs.⁴¹ The pharmacokinetics of HPCD are similar to that of inulin, and the toxic dose (nephrotoxicity) has been estimated to be 200 mg/kg in rats.⁴² The dose of HPCD used to dissolve 17-beta estradiol in the present study was 0.63 mg/kg, far below the toxic dose. Furthermore, HPCD has been used for administration of ophthalmic preparations and intravenous anaesthetic agents in humans.^{43,44} HPCD complexed to 17-beta estradiol has been used to enhance bioavailability of orally, or, sublingually administered 17-beta estradiol with no untoward effects in humans.⁴⁵

Retrospective studies in humans have shown no benefit of hormonal replacement therapy on angiographic restenosis following PTCA⁴⁶ although one study did show a beneficial effect after directional atherectomy.⁴⁷

5 However, it should be noted that conjugated estrogen (and not 17-beta estradiol) was the predominant form of estrogen used in many of these patients, and, no information about concomitant use of progesterone is available.

10 In conclusion, we have shown that, a single dose of 17-beta estradiol delivered locally during PTCA has the potential to inhibit neointima proliferation effectively. The delivery of 17-beta estradiol can be performed easily with the InfusaSleeve catheter, without risk of additional injury. With this approach, it may be possible to avoid potential undesirable effects of

15 long term systemic administration of estrogen. ER β has been identified in humans, and inhibition of proliferation of human vascular SMC by 17-beta estradiol has been demonstrated in cell culture assays. The local administration of 17-beta estradiol is therefore a promising new approach, which might be useful in preventing the proliferative response after PTCA in

20 humans. Its usefulness in preventing restenosis after PTCA is contemplative in view of the foregoing promising results.

Example 2: The effect of estradiol on vascular endothelial function

Methods

25 **Animal preparation**

The study protocol was approved by the Animal Care and Ethical Research Committee of the Montreal Heart Institute. Juvenile farm pigs weighing 20-25 kg (1 female, and 8 castrated males) were used. On the day of the

30 experiment, animals received 650 mg of acetylsalicylic acid and 30 mg of

nifedipine orally, were premedicated with 6mg/kg of tiletamine hydrochloride and zolazepam hydrochloride, and were given 0.05 mg of atropine intramuscularly. Under general anesthesia (a mixture of 1-1.5% isoflurane and oxygen enriched air), the right femoral artery was cannulated percutaneously. An 8 Fr arterial sheath was introduced, and 100 mg/kg of lidocaine and 250 U/kg of heparin were administered intra-arterially. Additional heparin was administered during PTCA if needed, to maintain an activated coagulation time of > 300 seconds.

10 Procedure

An 8 Fr right Amplatz guiding catheter and right Judkins guiding catheter were used for cannulation of the left and right coronary arteries, respectively. A standard balloon catheter (corresponding to a balloon/artery ratio of 1.1-1.3: 1) was advanced over a 0.014" floppy guide wire, and 3 successive 30-second inflations at 10 atm pressure were made with a 30 second interval between each inflation. PTCA was performed on all 3 coronary meries of each animal. For local delivery, the InfusaSleeve catheter (LocalMed Inc.) was used, which permits safe drug delivery with negligible additional injury (7). After balloon dilatation, each coronary artery of an animal was randomized to receive either 600 µg of 17-beta estradiol (in 5 ml), vehicle alone (5 ml), or PTCA only. The vehicle 2-hydroxypropyl-beta-cyclodextrin (HPCD), and 17-beta estradiol were obtained from Sigma Chem. Co. For local delivery with the InfusaSleeve catheter, a proximal driving pressure of 10 atm and support balloon pressure of 6 atm were utilized.

Intracoronary infusion

All 9 animals underwent cardiac catheterization at the end of 4 weeks. After a baseline coronary angiogram, selective cannulation of the proximal portion

of a coronary artery was performed with a single lumen balloon catheter (TotalCross, Schneider) for the administration of vasoactive agents. Acetylcholine (Ach) in increasing concentrations of 10^{-7} M, 10^{-6} M, 10^{-5} M, 10^{-4} M, was successively infused through the lumen port of the catheter.

- 5 Each dose was administered for a duration of 3 minutes at a constant rate of 1 ml/min using an infusion pump. Coronary angiography was performed at the end of each dose. After infusion of the highest concentration of Ach (10^{-4} M and angiography, 100 µg of nitroglycerin was administered via the lumen port of the catheter, and a coronary angiogram performed. The same
- 10 protocol was repeated for the other 2 coronary arteries. Heart rate, blood pressure, and ECG were monitored continuously throughout the experiment.

Quantitative coronary angiography

- 15 Coronary angiography was performed with a single plane imaging system (Electromed Intl). Images were obtained in predetermined views which best demonstrated the vessel segment of interest and without overlap of branches. Care was taken to maintain the same angulation during angiography of a segment throughout the procedure. Ionic contrast (MD-76, Mallinckrodt Medical Inc) was used throughout the experiment. Images were captured at a frame speed of 30 frames/sec, and stored digitally. A segment of contrast-filled guiding catheter was included in every frame, for the purpose of calibration. Calibration was performed using the known diameter of the contrast-filled guiding catheter as the reference segment, to avoid
- 20 error due to magnification. Coronary artery diameter measurements were made using a validated computerized edge-detection system (8). The midpoint of the injured segment was used for calculation of coronary artery diameter. For each analysis, coronary artery diameter measurements were performed in 3 consecutive end-diastolic frames, and the results averaged.
- 25

Measurements were performed by an independent observer blinded to the treatment group of the vessels.

Immunohistochemistry

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The animals were euthanized at 4 weeks. Under general anesthesia as described above, exsanguination was performed with replacement by 1 l of 0.9 % NaCl solution. The heart was perfusion-fixed *in vivo* with 2 l of 10 % buffered formalin at 200 mm Hg pressure. The heart was then removed, and 10 the coronary arteries were harvested immediately. From the injured segment (identified in relation to side branches), serial sections of 3-5 mm were made, and stored in 10 % buffered formalin solution. The sections were then treated with incremental concentrations of alcohol followed by treatment with xylene and paraffin. Slices of 6 µm thickness were prepared, and stained with 15 Verhoeff's stain for assessment of tissue response to injury. For each injured segment, 2 slices demonstrating maximal neointima response were selected for immunohistochemistry, and the results obtained from analysis of the cross sections were averaged. The % of reendothelialization and, the % of endothelial nitric oxide synthase (eNOS) expression were calculated as 20 follows: (the total length of the luminal surface staining positively / the perimeter of the lumen) x 100, respectively. Analysis was performed by an independent examiner with no knowledge of the treatment groups to which the sections belonged. For lectin immunohistochemistry, the 6 µm slices were first treated with hydrogen peroxide and methanol to block endogenous 25 peroxide, incubated with the *Dolichos biflorus* agglutinin (Sigma Chemical Co.) followed by treatment with 3,3'-diaminobenzidine (Vector Laboratories) and, subsequently counter-stained with hematoxylin. For immunohistochemistry of eNOS expression, after blocking of endogenous peroxide and non-specific antibodies, the slices were treated serially with the primary 30 mouse anti-eNOS antibody (Bio/Can Scientific), the secondary goat anti-

mouse antibody (Vector Laboratories), incubated with avidin-biotin (Vector Laboratories), treated with 3,3'-diaminobenzidine (Vector Laboratories) and finally counter-stained with hematoxylin. For both immunohistochemical examinations, normal porcine carotid artery slices were used as positive controls; whereas slices obtained from the injured coronary arteries and stained only with hematoxylin were used as negative controls.

Statistical analysis

Values are expressed as mean \pm SD. Comparison of basal coronary artery diameter among the 3 groups was made using the one-way analysis of variance test. Comparisons between basal coronary artery diameter and coronary artery diameter following infusion of vasoactive agents were made with two-tailed Student's t-tests. The Kruskal-Wallis test was used for comparison of lectin and eNOS expression among the 3 treatment groups. Linear relationships between lectin expression and response to Ach, and between eNOS expression and response to Ach were analyzed with Pearson correlation coefficients. Values were considered to be statistically significant if $p < 0.05$.

20

Results

There were no significant differences in basal coronary artery diameter (2.53 ± 0.6 mm for 17-beta estradiol, 2.79 ± 0.35 mm for PTCA only, and 25 2.77 ± 0.44 mm for vehicle groups respectively, $p < 0.4$) among the 3 treatment groups. The extent of morphologic tissue injury (9) among the groups was similar. No changes in heart rate, ECG, or blood pressure were noted during the local delivery or during intracoronary infusion of vasoactive agents.

30

Response of PTCA only group to Ach

Compared to the basal coronary artery diameter, there were no significant changes in coronary artery diameter following intracoronary infusion of 10^{-7} M and 10^{-6} M concentrations of Ach (Table). At a concentration of 10^{-4} M a significant vasoconstrictive response was noted ($p < 0.02$). A marked vasoconstrictive response was observed at a concentration of 10^{-4} M ($p < 0.0001$) (Figure 3). The vasoconstriction was completely reversed upon administration of the endothelium-independent vasodilator nitroglycerin.

Coronary diameter increased from 1.8 ± 0.48 mm after 10^{-4} M Ach, to 2.5 ± 0.28 mm following nitroglycerin ($p < 0.01$; $p = 0.2$ for post-nitroglycerin vs basal diameter).

Response of vehicle treatment group to Ach

Compared to the basal coronary artery diameter, 10^{-7} M Ach did not change coronary artery diameter in the vehicle treatment group (Table 3). A trend towards significant vasoconstriction was noted with 10^{-6} M Ach ($p = 0.06$). Significant vasoconstriction was produced by 10^{-5} M ($p < 0.02$), and at 10^{-4} M ($p < 0.001$) Ach infusion respectively (Figure 3). Nitroglycerin completely reversed the vasoconstriction, returning the arteries to their basal diameter (from 1.89 ± 0.51 mm after 10^{-4} M Ach, to 2.69 ± 0.52 mm following nitroglycerin [$p < 0.004$; $p = 0.7$ for post-nitroglycerin vs basal diameter]).

25 Response of 17-beta estradiol treated group to Ach

In the vessels treated with local delivery of 17-beta estradiol no significant vasoconstrictive response to Ach occurred at any concentration used (Table) (Figure 3). A mild and statistically nonsignificant increase in coronary artery diameter was observed following administration of nitroglycerin: from $2.28 \pm$

0.61 mm after 10^{-4} M Ach to 2.61 ± 0.48 mm after nitroglycerin ($p = 0.4$; $p = 0.8$ for post-nitroglycerin vs basal diameter).

Immunohistochemistry

5

Immunohistochemical analyses were performed 4 weeks after PTCA on all 9 animals. Three arterial segments were lost/damaged during harvesting of the samples (2 of PTCA only group, and 1 of vehicle group). Significant differences were seen among the 3 treatment groups in the extent of reendothelialization, as assessed by immunohistochemical analysis with the lectin *Dolichos biflorus* agglutinin (Figure 4). Reendothelialization was noted to a greater extent in vessels treated with local delivery of 17-beta estradiol compared to the other 2 groups (90.6 ± 5.5 % for 17-beta estradiol 71 ± 6.8 % for PTCA only, and 72.8 ± 4.9 % for vehicle, $p < 0.0005$).
10 Endothelial nitric oxide synthase expression was also higher in vessels treated with 17-beta estradiol (35.6 ± 11.8 % for 17-beta estradiol 9.4 ± 3.9 % for PTCA only, and 9.2 ± 4.0 % for vehicle, $p < 0.0005$) (Figure 5). No significant differences in immunohistochemical analyses were observed between vessels treated with vehicle or PTCA only.

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We proceeded further to analyze whether a linear relationship between reendothelialization and the response to Ach could be demonstrated. A significant inverse correlation was noted between reendothelialization as assessed by immunohistochemistry with the lectin *Dolichos biflorus* agglutinin and the response to Ach ($r = 0.48$, $p < 0.02$) (Figure 6). An even stronger inverse linear correlation was observed between eNOS expression and the response to Ach ($r = -0.58$, $p < 0.005$).

Conclusions

This study demonstrates for the first time that local delivery of 17-beta estradiol immediately following PTCA enhances subsequent 5 reendothelialization and endothelial function at the site of injury. Besides its critical role in the regulation of vascular tone, the normal endothelium functions as an effective barrier between blood elements and underlying vascular smooth muscle cells. Endothelium-derived nitric oxide (NO) is a potent vasodilator, inhibits monocyte adherence and platelet aggregation 10 and adhesion (10), vascular smooth muscle cell migration (11) and proliferation (12).

PTCA is associated with arterial injury and damage to the endothelium (3). Following arterial injury, varying rates of reendothelialization have been 15 reported. Reendothelialization rates of 81 % (13), and even lower rates of < 50 % (14) following arterial injury have been observed. In a study of specimens of restenotic lesions obtained by atherectomy in humans, no endothelial cells could be demonstrated (15). In the present study, local treatment with 17-beta estradiol was followed by nearly complete 20 reendothelialization ($90.6 \pm 5.5\%$), which was significantly greater than that observed in the groups not treated with 17-beta estradiol. Estrogen receptors have been identified in human coronary artery and umbilical vein endothelial cells (16), and when bound to estrogen are capable of regulating protein synthesis by altering transcription rates (17). In cell culture assay of human 25 umbilical vein endothelial cells, treatment with 17-beta estradiol markedly increased both cell migration and proliferation (18). Therapy with subcutaneously implanted 17-beta estradiol pellets significantly enhanced reendothelialization following arterial injury (6). The capacity of 17-beta estradiol to increase vascular endothelial growth factor synthesis (19) and 30 the effect of 17-beta estradiol on basic fibroblast growth factor may be

responsible for the enhanced reendothelialization. Vascular endothelial growth factor treatment is known to promote reendothelialization *in vivo* (20). In human umbilical vein and coronary artery endothelial cell culture experiments, treatment with 17-beta estradiol enhanced the release and phosphorylation of basic fibroblast growth factor (21,22). It has been shown that administration of basic fibroblast growth factor *in vivo* stimulates reendothelialization following arterial injury in rats (23). Another mechanism by which 17-beta estradiol could possibly influence extent of reendothelialization is by inhibition of apoptosis of injured endothelial cells: a 50 % decrease in apoptosis was seen with 17-beta estradiol treatment of human umbilical vein endothelial cells exposed to tumor necrosis factor- α (24). It is noteworthy that increased expression of tumor necrosis factors is known to occur following balloon injury (25)-

Impaired endothelial function, as in atherosclerosis (26) or following experimental inhibition of NO (27), has been associated with a paradoxical constrictive response to Ach. This paradoxical response to Ach could be modified by treatment with estrogen. In humans, 17-beta estradiol administered intravenously (28) or by continuous intracoronary infusion (29), attenuated the vasoconstrictive response to Ach and also inhibited the Ach-induced increase in coronary resistance and decrease in coronary blood flow. The regulatory effect of 17-beta estradiol on eNOS that we observed may be responsible for the beneficial effects on endothelial function, as vascular response to Ach is closely related to eNOS expression (30,31). In support of this notion, a strong inverse linear relationship was seen between the vascular response to Ach and eNOS expression (Figure 4). The ability of estrogen to induce nitric oxide synthase was first identified during gestation in guinea pigs (32). Induction of eNOS function by 17-beta estradiol has been subsequently demonstrated to be accompanied by increased eNOS protein and mRNA expression (33,34). Increased circulating NO levels

have been observed in postmenopausal women treated with 17-beta estradiol (35). Following arterial injury, the regenerated endothelium is often functionally abnormal (5). Abnormal vasomotion as a result of persistent endothelial dysfunction at the site of angioplasty has been demonstrated in

5 patients undergoing PTCA, and is postulated to be responsible for the symptom of angina noted in patients with nonsignificant stenosis following PTCA (36). We have shown that functional abnormalities could be improved significantly by treatment with locally delivered 17-beta estradiol. A unifying hypothesis for the responses we observed is that eNOS downregulation

10 following PTCA prevents the vasodilatory response to Ach mediated by endothelial NO production. By improving eNOS expression, 17-beta estradiol allows the vasodilatory response of Ach to counteract its direct vasoconstricting action, preventing Ach-induced vasoconstriction at the site of local injury. The vasodilatory response to nitroglycerin in Ach-constricted

15 arteries post-PTCA is consistent with this concept, since exogenous nitroglycerin (which is a NO donor) simply provides a local NO-related dilation that the eNOS deficient angioplastied segment cannot provide for itself.

20 Both rapid non-genomic and genomic effects have been postulated to be involved in the influence of 17-beta estradiol on coronary vasculature (37,38). Although increased protein synthesis was not quantified in the present study, the enhanced eNOS expression and the response to Ach observed as late as 28 days following a single dose of 17-beta estradiol

25 appears to be consistent with a genomic effect. This is the first study to suggest the existence of a genomic effect following local therapy with 17-beta estradiol in coronary circulation *in vivo*.

30 Gender differences in the endothelium-dependent vasodilation by 17-beta estradiol have been noted (39). In our study, a majority of animals were

mates and a significant beneficial effect of 17-beta estradiol was noted in all the animals studied, irrespective of sex. Thus, local delivery of 17-beta estradiol appears to be effective in males as well as females. There is evidence to suggest that the simultaneous administration of progesterone 5 reduces NO levels induced by 17-beta estradiol (35), this issue was, however, beyond the scope of the present study.

We conclude that a single dose of 17-beta estradiol delivered locally following balloon injury can significantly improve reendothelialization and 10 enhance endothelial function at the injured site as late as 1 month following injury. Besides the beneficial vascular effects of improved endothelial function, this observation may be of particular importance following balloon angioplasty as improved endothelial function is known to be associated with decreased neointima formation in the injured area (20,40). This approach 15 merits further study, with a view to potential clinical value in the prevention of vascular dysfunction and restenosis following PTCA.

Formulations

20 The formulations may include estradiol or a derivative thereof and any pharmaceutically acceptable vehicle. Since estradiol is a lipophilic molecule, such vehicle would ideally include a solvent component. Such a solvent component includes molecules such as propylene glycol, ethanol, and detergents, for example Pluronics™. The formulations may take the form of 25 a liquid, a suspension, a semi-solid or a thermoreversible composition which may form a layer over the endothelium. The formulations may further be included in or used as a coating for a device such as a stent, or be part of any similar device that can be left *in-situ* upon angioplasty or vascular surgery.

Although the present invention has been described hereinabove by way of preferred embodiments thereof, these embodiments can be modified at will, without departing from the spirit and nature of the subject invention. Such modifications are within the scope of the present invention as defined in the
5 appended claims.

Table 1: Morphometric Analysis

Characteristics	17-beta estradiol	PTCA only	Vehicle alone	p value*
Segments analyzed	12	9	10	NS
Artery size (mm)	2.86 ± 0.35	2.94 ± 0.24	2.94 ± 0.41	NS
Balloon/Artery ratio	1.22 ± 0.09	1.2 ± 0.06	1.17 ± 0.11	NS
EEL _{ref} /EEL _{inj} †	1.01 ± 0.16	1.31 ± 0.37	1.16 ± 0.28	NS
Neointima area (mm ²)	0.4 ± 0.3	0.88 ± 0.61	1.14 ± 1.03	< 0.05
% neointima	12.16 ± 8.89	23.02 ± 11.91	25.46 ± 14.96	< 0.025
Neointima/Media area	0.59 ± 0.48	1.67 ± 1.29	1.75 ± 1.29	< 0.01
% stenosis	15.67 ± 11.13	27.51 ± 13.17	30.34 ± 17.05	< 0.025
Restenotic index	1.3 ± 0.5	2.4 ± 0.68	2.42 ± 0.71	< 0.005
Injury score	1.64 ± 0.34	1.7 ± 0.43	1.77 ± 0.47	NS

* 17-beta estradiol vs other 2 groups; †EEL_{ref} = proximal reference segment external elastic lamina area, EEL_{inj} = injured segment external elastic lamina area (averaged).

Table 2: Response to 17-beta estradiol According to Sex of the Animal

Characteristics	Male	Female	p value
Restenotic index	1.2 ± 0.59	1.37 ± 0.45	> 0.1
Neointima area (mm ²)	0.51 ± 0.34	0.25 ± 0.15	> 0.1
Neointima/Media area	0.78 ± 0.55	0.32 ± 0.16	> 0.1
% neointima	14.93 ± 10.68	8.29 ± 3.72	> 0.1
% stenosis	18.93 ± 13.39	11.09 ± 5.16	> 0.1

Table 3: Response to Intracoronary Acetylcholine

Ach*	Diameter-basal (mm)	Diameter-post Ach (mm)	p value
<i>PTCA group</i>			
10 ⁻⁷ M	2.79 ± 0.35	2.65 ± 0.35	0.4
10 ⁻⁶ M	2.79 ± 0.35	2.54 ± 0.32	0.1
10 ⁻⁵ M	2.79 ± 0.35	2.3 ± 0.35	0.02
10 ⁻⁴ M	2.79 ± 0.35	1.8 ± 0.48	0.0001
<i>Vehicle group</i>			
10 ⁻⁷ M	2.77 ± 0.44	2.6 ± 0.41	0.4
10 ⁻⁶ M	2.77 ± 0.44	2.33 ± 0.5	0.06
10 ⁻⁵ M	2.77 ± 0.44	2.24 ± 0.47	0.02
10 ⁻⁴ M	2.77 ± 0.44	1.89 ± 0.51	0.001
<i>17-beta estradiol group</i>			
10 ⁻⁷ M	2.53 ± 0.6	2.46 ± 0.58	0.8
10 ⁻⁶ M	2.53 ± 0.6	2.38 ± 0.58	0.6
10 ⁻⁵ M	2.53 ± 0.6	2.36 ± 0.59	0.6
10 ⁻⁴ M	2.53 ± 0.6	2.28 ± 0.61	0.4

* acetylcholine

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Amended 34

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WHAT IS CLAIMED IS:

1. The use of 17- β estradiol or a derivative thereof in the making of a medication or a device for in-situ administration in the lumen of a blood vessel having suffered vascular injury, at the injured site, for improving reendothelialization and vascular endothelial function in a patient.

2. The use as defined in claim 1, wherein 17- β estradiol or a derivative thereof is present in a dose unit of 1 to 5000 $\mu\text{g}/\text{Kg}$ of patient's body weight.

3. The use, as defined in claim 1, wherein 17- β estradiol or a derivative thereof is present in a dose unit of 10 to 50 $\mu\text{g}/\text{Kg}$ of patient's body weight.

4. The use as defined in claim 1, wherein 17- β estradiol or a derivative thereof is present in a dose unit of 10 to 30 $\mu\text{g}/\text{Kg}$ of patient's body weight.

5. The use as defined in any one of claims 1 to 4, wherein said pharmaceutically acceptable carrier comprises hydroxypropyl-beta-cyclodextrin (HPCD).

6. The use as defined in claim 5, wherein HPCD is present in a dose capable of solubilizing 17-beta estradiol or a derivative thereof.

7. The use as defined in Claim 4, where 17-beta-estradiol or a derivative thereof is admixed with a carrier comprising at

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least 0.63 mg hydroxypropyl-beta-cyclodextrin per kilogram of patient's body weight.

8. The use as defined in any one of claims 1 to 7, which is for a single administration. ← new

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(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

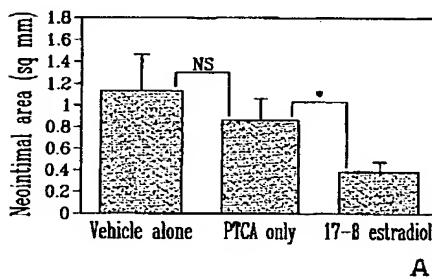
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Published:

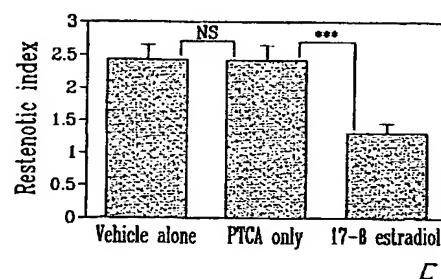
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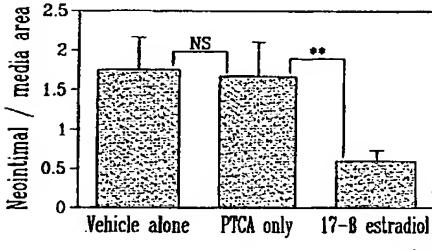
(54) Title: LOCAL DELIVERY OF 17-BETA ESTRADIOL FOR PREVENTING VASCULAR INTIMA HYPERPLASIA AND FOR IMPROVING VASCULAR ENDOTHELIUM FUNCTION AFTER VASCULAR INJURY



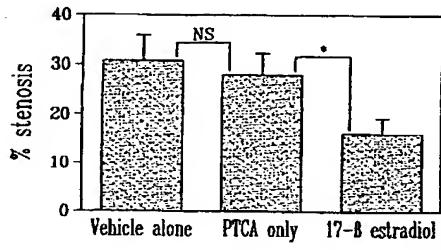
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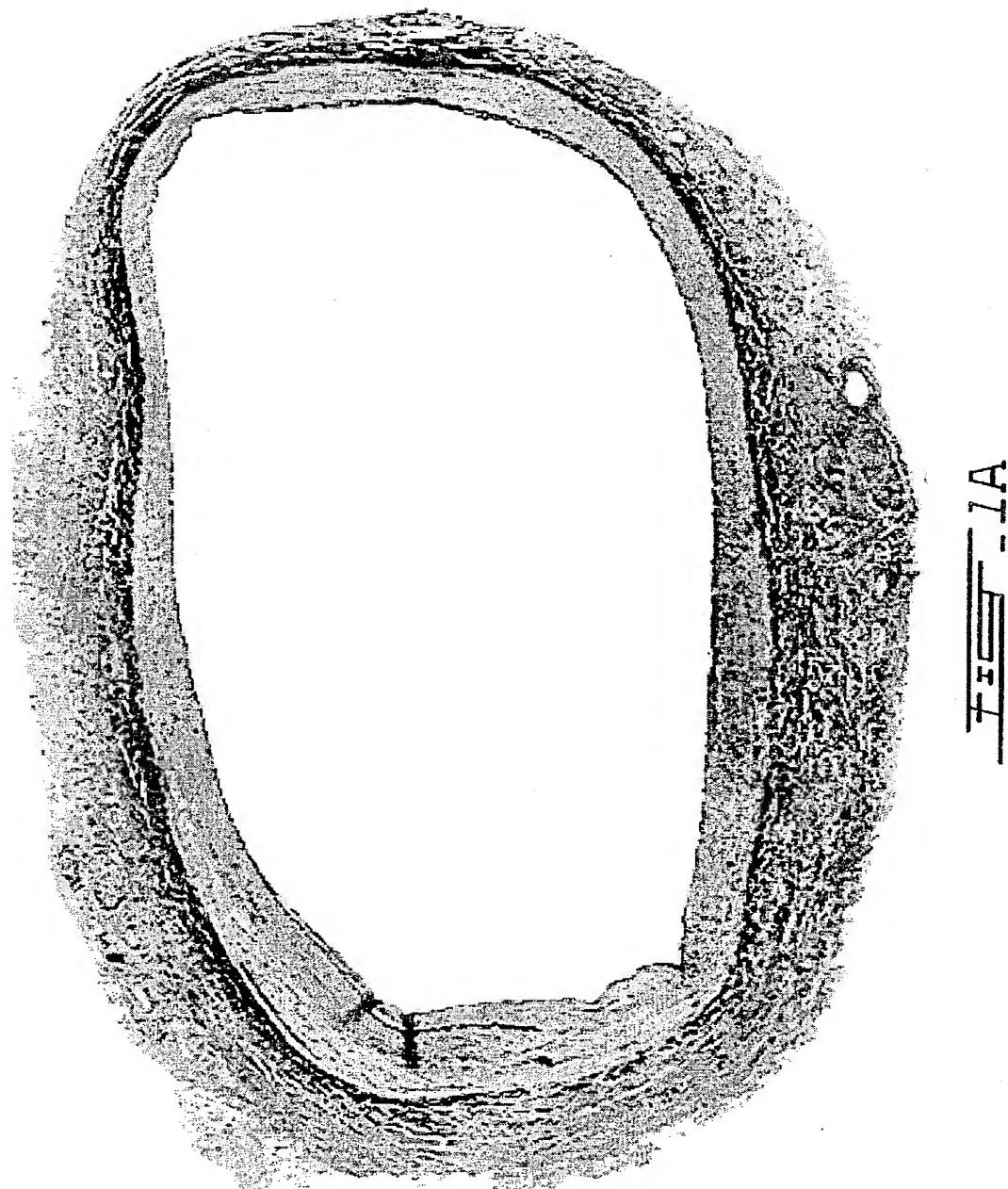
(57) Abstract: The cardioprotective effects of estrogen are well recognized. In *in vitro* experiments, and upon systemic administration, 17-beta estradiol has shown to inhibit vascular smooth muscle cell proliferation and intima hyperplasia and to improve vascular endothelium function, after vascular injury. We hypothesized that locally delivered 17-beta estradiol could prevent restenosis. Compositions are used of 17-beta estradiol for *in-situ* administration at a vascular injured site are objects of the present invention.

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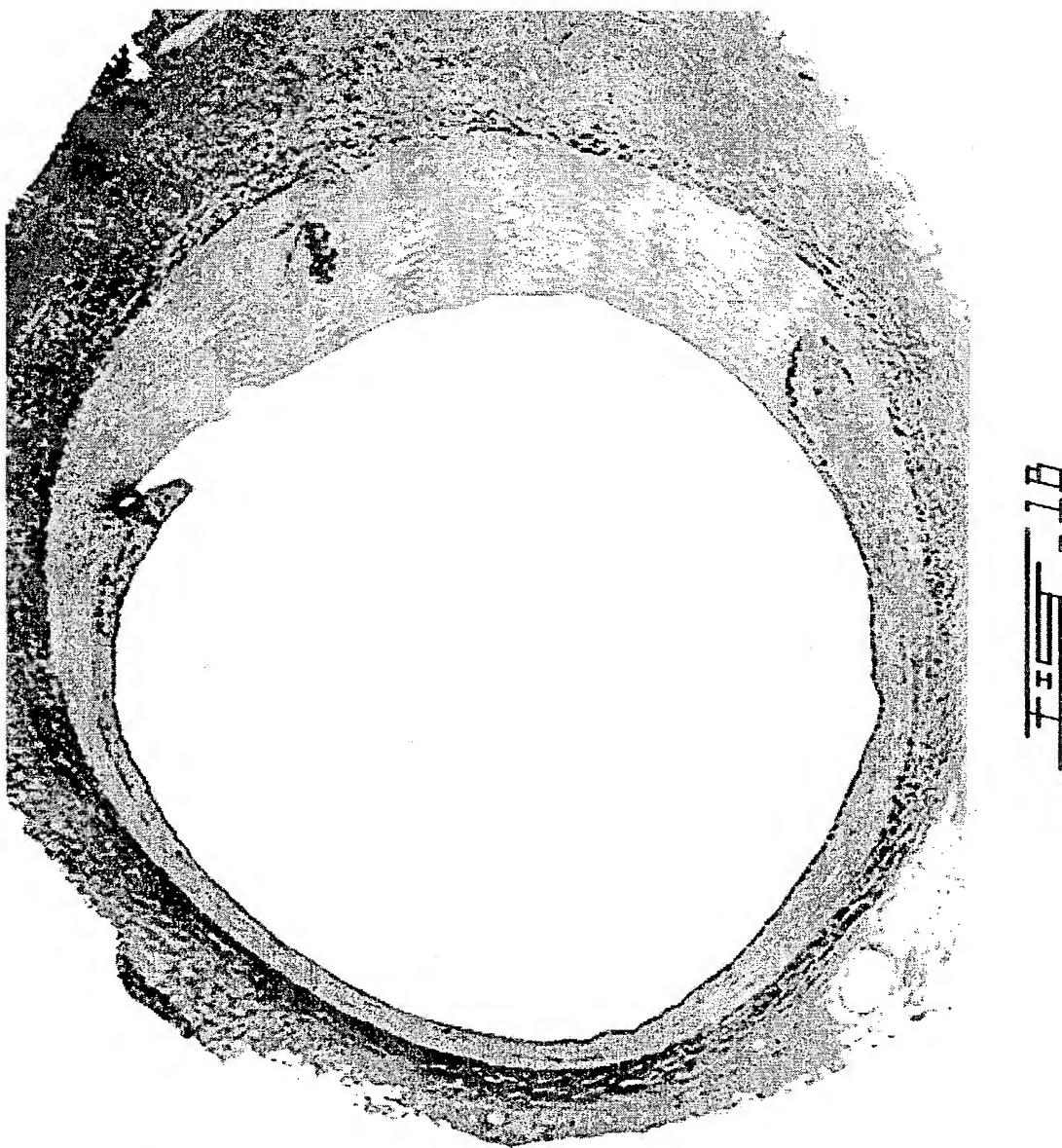


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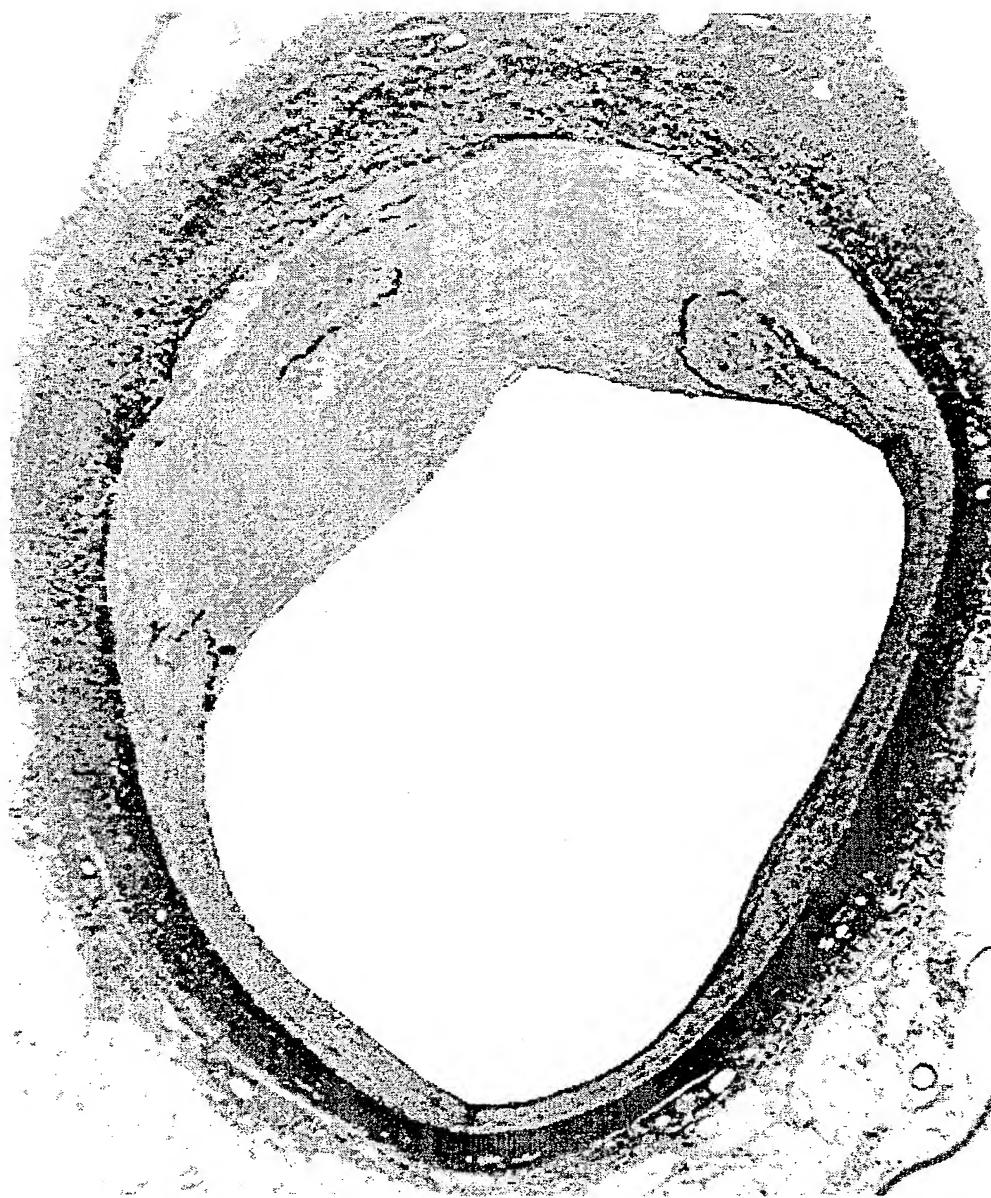


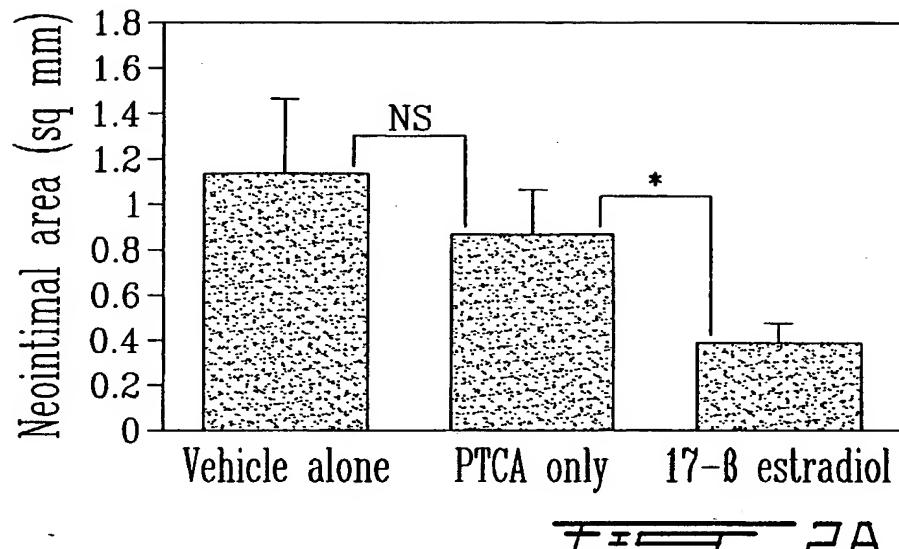
FIG - 1C

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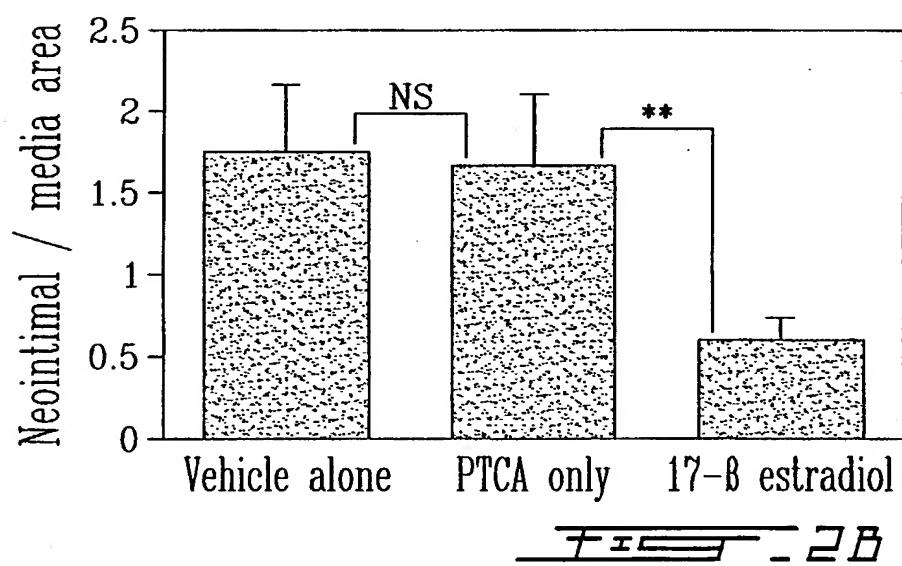
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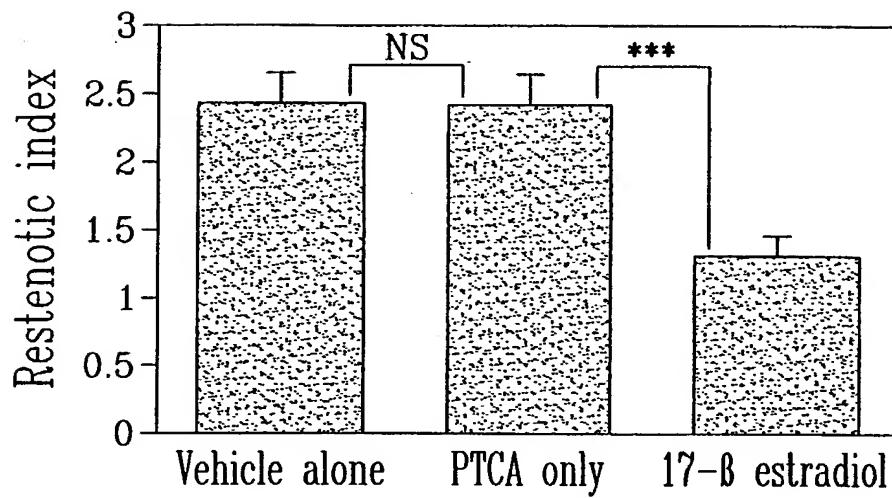
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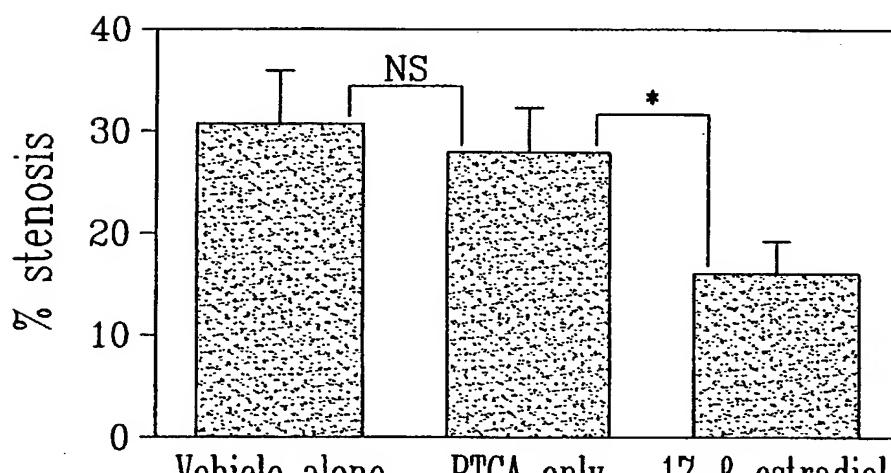
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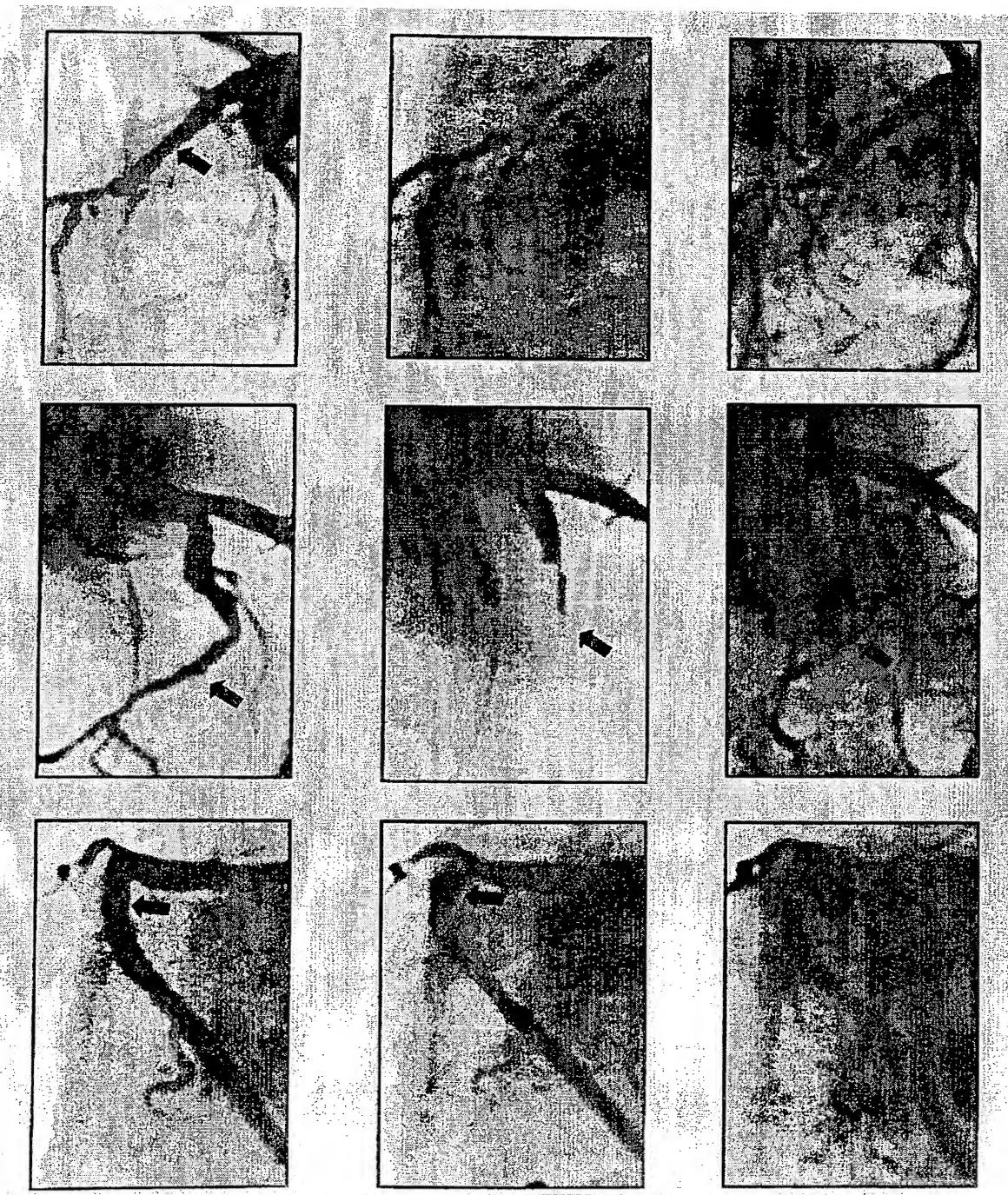


FIG - 3A

FIG - 3B

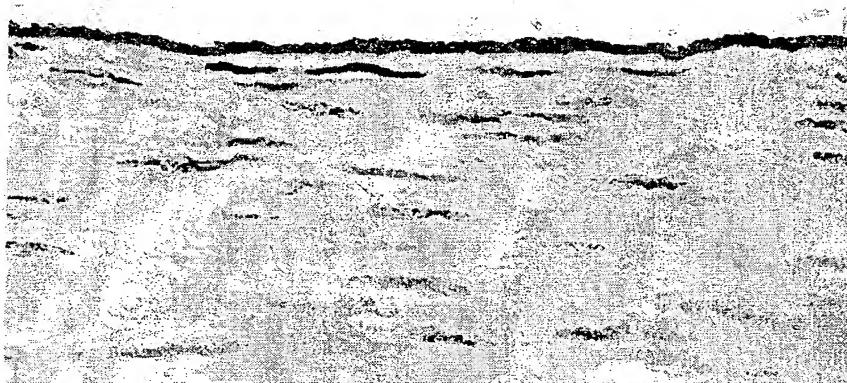
FIG - 3C

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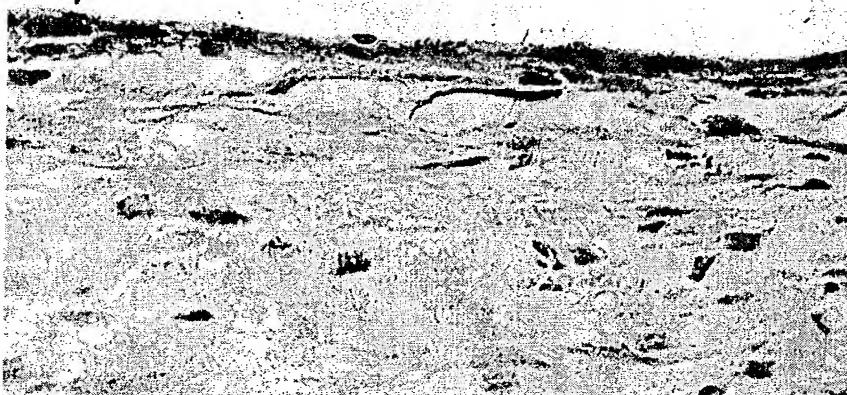
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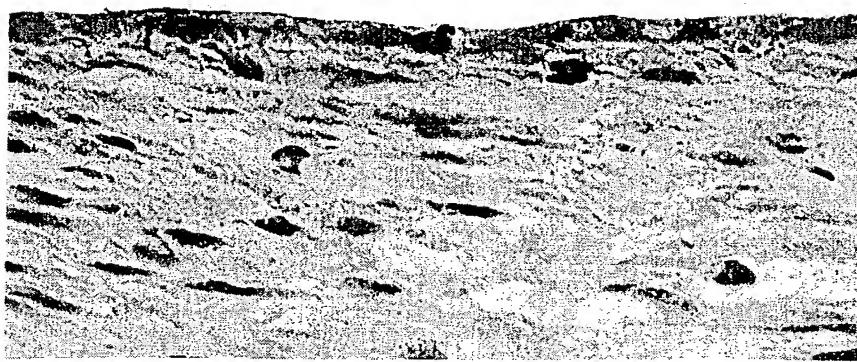
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~~F=~~ - 4A



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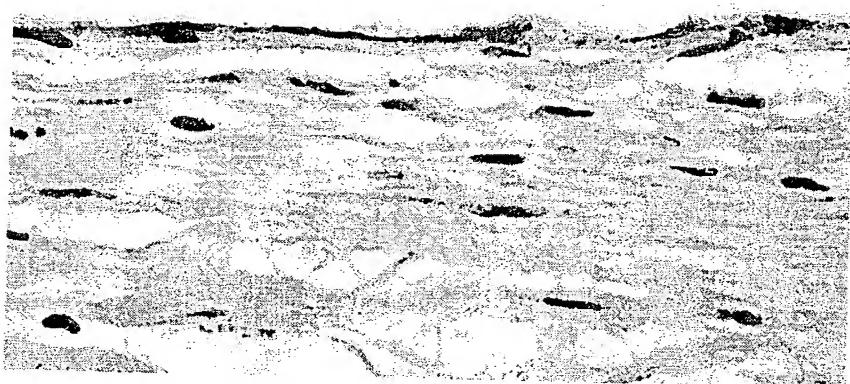
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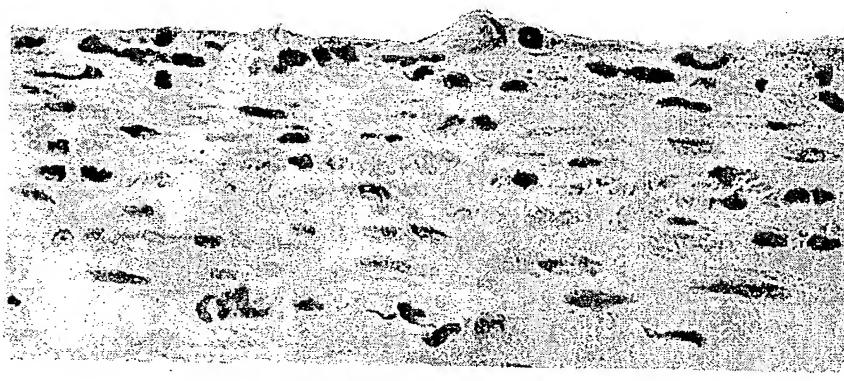
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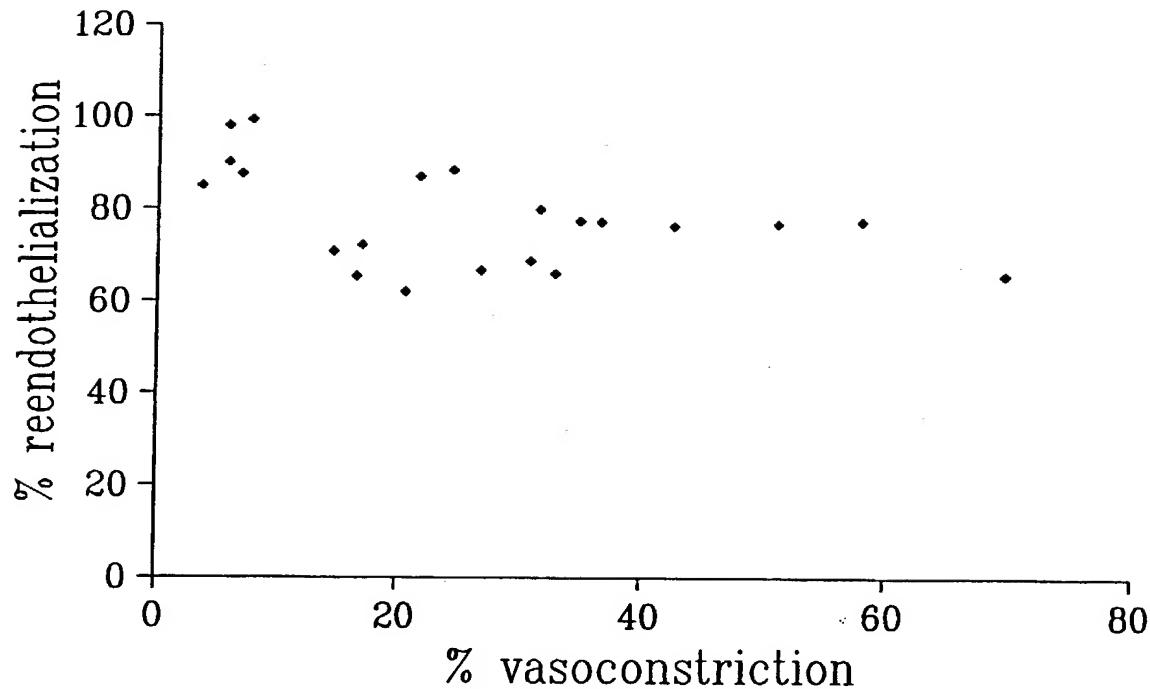
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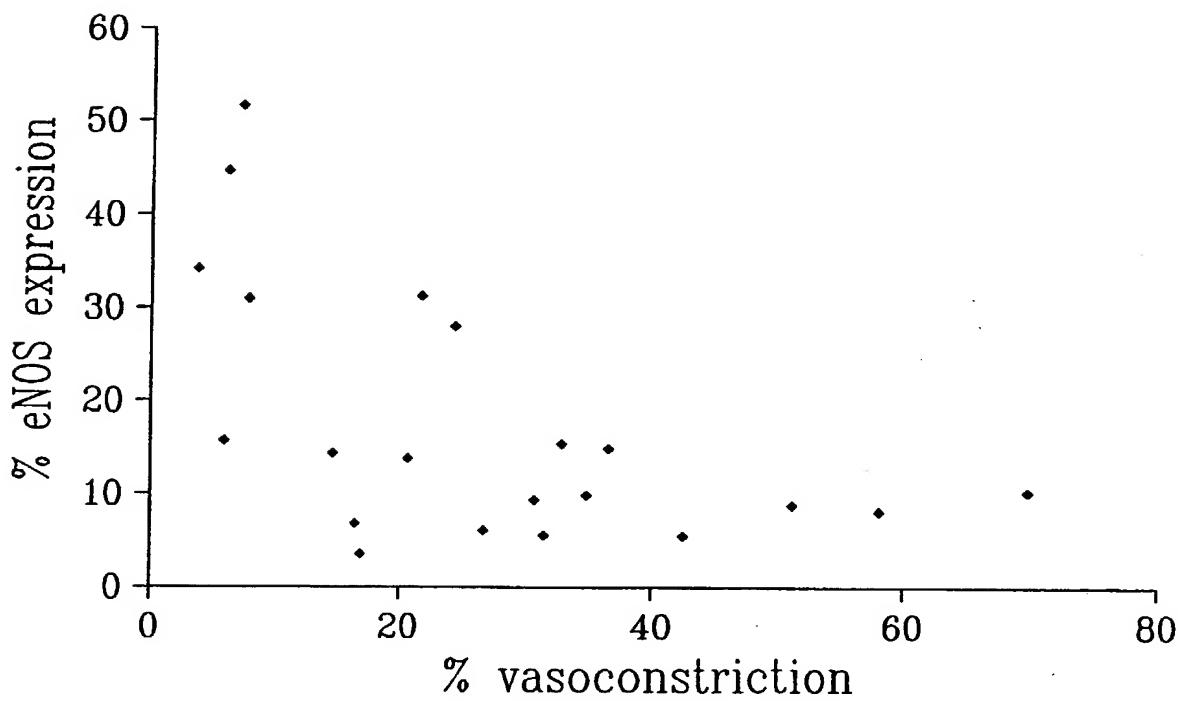
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~~7-8~~ - 6A



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US

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY
(Includes Reference to PCT International Applications)

ATTORNEY DOCKET
NUMBER
410718.90395

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

LOCAL DELIVERY OF 17-BETA ESTRADIOL FOR PREVENTING VASCULAR INTIMA HYPERPLASIA AND FOR IMPROVING VASCULAR ENDOTHELIUM FUNCTION AFTER VASCULAR INJURY

the specification of which (check only one item below):

is attached hereto.

was filed as U.S. Patent Application Serial Number _____
on ___,
as amended on ____ (if applicable).

was filed as a PCT international application number PCT/CA00/01132 on 21 Sept 2000
as amended under PCT Article 19 on 11/19/01 (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the applications for which priority is claimed:

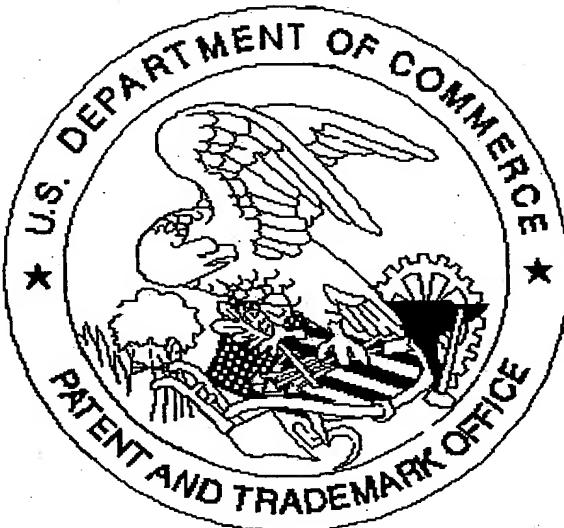
PRIOR FOREIGN PATENT APPLICATION(S) AND ANY PRIORITY CLAIMED UNDER 35 U.S.C. §119:

COUNTRY (If PCT Indicate PCT)	APPLICATION NUMBER	DATE OF FILING (Day, Month, Year)	PRIORITY CLAIMED UNDER 35 USC 119
PCT	PCT/CA00/01132	21 Sept 2000	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
CA	2,282,982	21 Sept 1999	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
CA	2,300,246	09 March 2000	<input checked="" type="checkbox"/> YES <input type="checkbox"/> NO
			<input type="checkbox"/> YES <input type="checkbox"/> NO

US

COMBINED DECLARATION FOR PATENT APPLICATION AND POWER OF ATTORNEY (Includes Reference to PCT International Applications)					ATTORNEY DOCKET NUMBER 410178.90395
I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in that/those prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application.					
PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. 120:					
U.S. APPLICATIONS			STATUS (Check One)		
U.S. APPLICATION NUMBER	U.S. FILING DATE		PATENTED	ABANDONED	PENDING
PCT APPLICATIONS DESIGNATING THE U.S.					
PCT APPLICATION NUMBER	PCT FILING DATE	U.S. SERIAL NUMBERS			
POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the U.S. Patent and Trademark Office connected therewith (List names and registration numbers):					
Send Correspondence to: <u>Jean C. Baker</u> <u>Quarles & Brady LLP</u> <u>411 East Wisconsin Ave. Suite 2550</u> <u>Milwaukee, WI 53202-4497</u>			Direct Telephone Calls to: <u>(414) 277-5000</u>		
201	FULL NAME OF INVENTOR	FAMILY NAME <u>CHANDRASEKAR</u>		FIRST GIVEN NAME <u>Baskaran</u>	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY <u>Dr. Radhakrishnan Nagar</u>		STATE OR COUNTRY <u>Chennai</u>	COUNTRY OF CITIZENSHIP <u>India</u>
	POST OFFICE ADDRESS	POST OFFICE ADDRESS <u>2, Justice Ramanujam Road</u>		CITY <u>Dr. Radhakrishnan Nagar, Chennai</u>	STATE & ZIP CODE/COUNTRY <u>India 600 041</u>
202	FULL NAME OF INVENTOR	FAMILY NAME <u>TANGUAY</u>		FIRST GIVEN NAME <u>Jean-Francois</u>	SECOND GIVEN NAME
	RESIDENCE & CITIZENSHIP	CITY <u>Montreal</u>		STATE OR COUNTRY <u>Quebec</u>	COUNTRY OF CITIZENSHIP <u>Canada</u>
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.					
SIGNATURE OF INVENTOR 201		SIGNATURE OF INVENTOR 202		SIGNATURE OF INVENTOR 203	
DATE <u>29/5/2002</u>	DATE <u>29/5/2002</u>	DATE <u>29/5/2002</u>	DATE	DATE	DATE

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